FACTORS INFLUENCING THE DEVELOPMENT AND TIME OF APPEARANCE OF MAMMARY CANCER IN THE RAT IN RESPONSE TO ESTROGEN

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The experimental production of mammary cancer by administration of estrogens (first accomplished in mice by Lacassagne [1] in 1932) indicated that a substance normally produced in the body and possessing physiologic function could lead to cancer. This method has made it possible to reproduce in laboratory animals (mice and rats) mammary cancers which apparently arise under circumstances similar to the natural conditions that bring about human cancer. It is important, therefore, to inquire into the mechanism by which cancer is thus produced and to compare it with the processes in human cancer.

EFFECT OF ESTROGENIC STIMULATION ON THE AGE OF THE MAMMARY GLAND

The primary effect of estrogen on the mammary gland is to accelerate the development of the duct tree and its supporting fibrous tissue. This is accompanied by ripening or enlargement in the mammary epithelium as well as in the cells of the stroma and addition of new elements through multiplication of cells. If accelerated development and ripening are maintained by continuous and intense estrogenic stimulation, the regeneration of tissue which supplies adult cells may ultimately be exhausted. Under these conditions aging and degeneration occur. In such a senescent gland, with further stimulation both benign and malignant tumors form.

Apparently susceptibility to cancer increases with the physiologic age of the breast rather than with the chronologic age of the animal. Repeated experiments have shown that the maximum size of the breast is attained sooner, but cannot be made to exceed by any significant degree that of the normal adult, by estrogenic stimulation. Increasing the

This investigation was aided by a grant from the Dr. Simon Baruch Foundation.

Read before the Section on Pathology and Physiology at the Ninety-Second Annual Meeting of the American Medical Association in Cleveland, June 5, 1941.

1. Lacassagne, A.: Compt. rend. Acad. d. sc. **195**:630, 1932.

amount or the duration of the stimulus beyond physiologic limits can therefore only accentuate maturity and aging in the mammary structures. This brings about pathologic changes which may terminate in

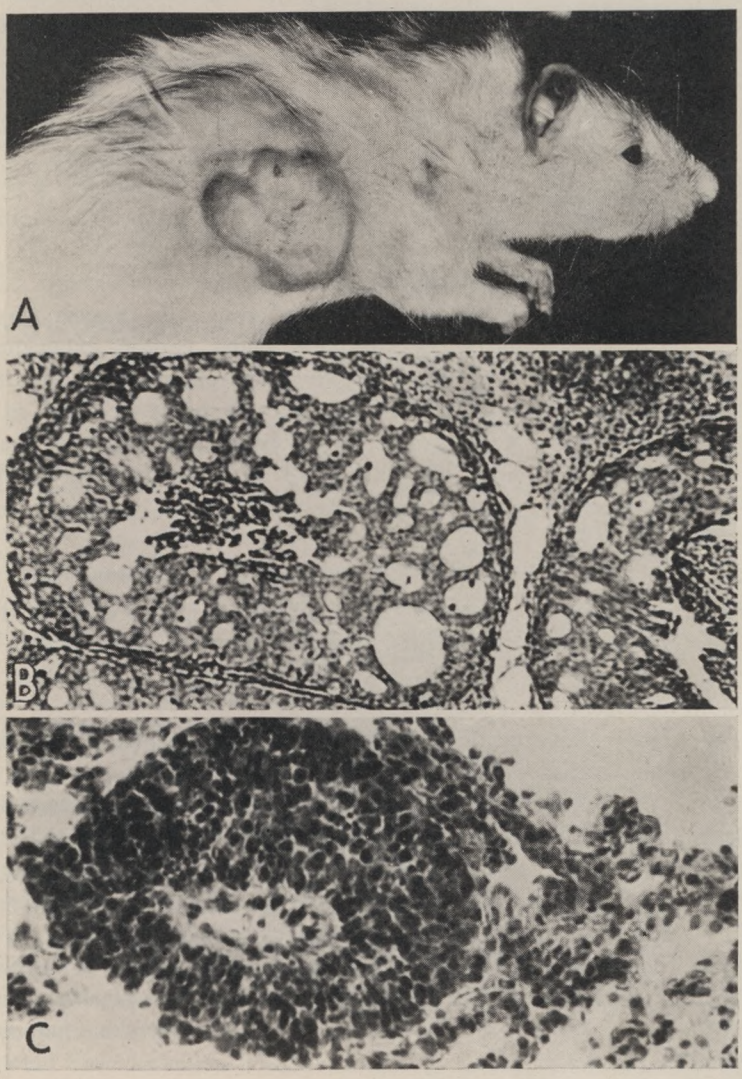

Fig. 1.—Estrogenic mammary cancer: *A*, the appearance of the tumor; *B*, its microscopic structure; *C*, a metastasis to the lung.

cancer. These end stages of estrogenic stimulation occur more quickly if the estrogen is administered in extremely high doses instead of moderately excessive amounts, or if it acts continuously (by dissolution

of implanted pellets)[2] rather than intermittently (by injection of a solution). The essential feature in the estrogenic production of mammary cancer, therefore, is the acceleration or the prolongation of ripening and maturity in the mammary gland beyond physiologic limits. If this condition is satisfied, cancer appears in the breasts of rats which are otherwise immune to the disease, whether these animals are young or old ones, males or females, castrates or noncastrates (fig. 1).

At the present writing, mammary cancers have occurred in 202 of 555 rats of an albino strain which were treated with estrogens. These animals have been maintained on a standard diet and inbred for a period of seven years, and under these conditions no spontaneous mammary cancer has been observed in a colony of over 5,000 animals. The percentage of rats in which estrogenic mammary cancers develop varies, but when the animals survive the required time, the disease may be present in 100 per cent of the survivors under certain experimental conditions. The majority of the experiments herein reported have been carried out in order to learn the time and the mode of onset of the cancers and only a few animals have been allowed to survive with macroscopic growths. Among these, only 2 animals showed multiple metastases to the lungs and lymph nodes.

INTENSITY AND DURATION OF ACTION OF ESTROGENS

A number of estrogenic compounds that vary in potency and in duration of effect may be used for the production of mammary cancer. The potency of each of these compounds and the length of its action are indicated in table 1.

The minimum amount of estrogen necessary to produce a physiologic reaction (cornification of the vaginal smear) in 50 per cent or more of a group of castrated female rats is spoken of as a rat unit and varies from a fraction to several micrograms according to the estrogen and the strain of rats used. If the estrogen has no prolonged action, the rat stays in estrus[3] for two to four days. With prolonged action, estrus may last weeks or months. In the normal rat, estrus usually lasts four to five days, and the amount of estrogen present varies from a fraction to several micrograms. As little as 3 to 5 micrograms of estrone (theelin) administered daily by injection over prolonged periods will produce pathologic changes.

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2. This statement holds for estrogens if one compares in the same group estrogens without a prolonged effect.

3. The term "estrus" refers to a group of anatomic and physiologic changes in the sexual organs. The uterus is enlarged and its lining thickened. The breasts also increase in size, and the lining of the mammary ducts is thickened. In the vagina, a similar thickening of the mucous membrane is accompanied by the shedding of ripened epithelial cells, and hence the appearance of cornified desquamated cells in the vaginal smear is taken as an index of estrus.

In woman estrus is roughly the time between two menstrual periods. Shorr and Cohen,[4] from observations on castrate women, estimated that from 3,000 to 5,000 micrograms of estradiol benzoate a day is necessary to produce human estrus. In woman, therefore, estrus lasts four to five times as long as in the rat and requires five thousand to ten thousand times as much hormone daily.

In considering the mechanism of mammary cancer it is important to bear in mind the ratios between physiologic and pathologic doses of estrogen and the ratio between the dosage for rats and that for man. As will be shown subsequently, mammary cancer results in rats within a period of one hundred and twenty days (which is approximately thirty times the length of normal estrus) when the amount of estradiol benzoate

TABLE 1.—*Potency and Period of Action of Various Estrogenic Compounds*

Compound	Potency in Millions of Rat Units per Gm.		Authors' Assays	Prolonged Action
	*	†		
Estradiol (alpha)	12	0.5	3.3	No
Estradiol (benzoate)	6	...	1.7	Yes
Estradiol (dipropionate)	5	...	2.5	Yes
Estrone (theelin)	1	0.3	1.0	No
Stilbestrol	3	0.6	3.3	No
Stilbestrol monomethyl ether	..	...	0.4	Yes
Stilbestrol dimethyl ether	..	...	0.04	Yes

* The figures in this column were obtained from the following sources: those for estradiol (alpha benzoate) and estrone from B. Whitman, O. Wintersteiner and E. Schwenk (J. Biol. Chem. **118**: 789, 1937); those for estradiol dipropionate from K. Miescher, C. Scholz and E. Tschopp (Biochem. J. **32**: 725, 1938); those for stilbestrol from E. C. Dodds, L. Golberg, W. Lawson and R. Robinson (Nature, London **142**: 34, 1938).

† These figures are from C. W. Sondern and J. L. Sealey (Endocrinology **27**: 670, 1940).

administered daily is approximately ten to fifteen times the physiologic dose. If the same factors are applied to the human breast, the length of time would approximate ninety weeks and the daily dose of the hormone would approximate 30 to 75 mg. This assumption has only theoretic interest since there is at present no way to evaluate the matter of species differences. Thus in experiments carried out by us on rabbits and monkeys cancers have failed to appear when corresponding amounts and periods of estrogenic stimulation were employed.[4a] Moreover, the calculated dose for man is based on the assumption that the subject is young or in the period of sexual maturity and that all of the organs are

4. Shorr, E., and Cohen, E. J.: Proc. Soc. Exper. Biol. & Med. **46**:330, 1941.

4a. Mammary cancer recently appeared in 1 rabbit after twenty months (six hundred and fifteen days) of treatment with stilbestrol dimethyl ether (in the dosage of 0.5 mg. daily). Cancers have failed to develop to date in a group of 6 monkeys stimulated continuously for more than three years with estrone and estradiol compounds implanted as pellets or injected (the total dose of estrogen exceeding 3 Gm. per monkey).

in their normal state. There are conditions (as will be pointed out subsequently) in which the cancer-producing threshold is greatly lowered.

There are two proofs that estrogens exert physiologic rather than direct chemical action in producing mammary cancer. One is that the cancer does not occur at the site of injection but appears, instead, in the organ (the breast) which the hormone influences physiologically. The second is that the periods of time required to produce cancer with estrogens of varying chemical composition are proportional to the physiologic potencies and independent of the chemical formulas (table 2 B and D). Therefore the estrogens cannot be considered chemically as cancerogenic agents.

Cancer occurs in the rat's breast when the estrogenic stimulation is (*a*) abnormally intensified, (*b*) prolonged and continuous or (*c*) augmented by other influences, such as previous endocrine disturbances. Intense interrupted stimulation is not as efficient as continuous estrogenic action obtained by the implantation of pellets or by the use of estrogens with prolonged action.

Relation of Intensity of Stimulation to Time of Appearance of Mammary Cancer.—The total amount of estrogen necessary to produce cancer is the same regardless of the daily dose administered if animals of the same age are treated by injection of estrone in oil. Thus it can be shown that with daily injection of estrone in oil the time required to produce mammary cancer in the rat is inversely proportional to the dose but that the total amount remains fairly constant (30 to 40 mg. of estrone). This was found true if daily doses of 50 to 200 micrograms were injected. It is possible that with daily doses beyond 200 micrograms of estrone increased excretion or destruction would occur and the time required for the appearance of the cancer would not be lessened.

Mammary cancer results when estrone in oil is injected subcutaneously in the backs of rats in daily doses above 0.025 mg. (25 micrograms). The cancers affect males and females, castrates and noncastrates. The cancers appear in five hundred to six hundred days when 50 micrograms is injected daily, within three hundred and fifty to four hundred days with 100 micrograms and within one hundred and fifty to two hundred days with 200 micrograms (table 2 A, B and C).

The relation of the intensity of stimulation to the time of appearance of mammary cancer may be demonstrated in another way by comparing the periods of time required to produce cancers with estrogens of varying potency. The time required varies in accordance with the physiologic strength of the compounds. The most potent estrogen will produce cancer in the briefest time [2] (table 2 D).

Effect of Interrupted Doses (Daily Injections of Estrone in Oil).—Because of the rapid ripening of the lobular buds when large amounts of estrogen are injected daily, immense dilated acinar structures or cysts

TABLE 2.—*Incidence and Time of Occurrence of Mammary Cancers in Rats Receiving Varying Doses of Estrogen in Oil*

Rat	Sex	Age	Castrated	Days Treated	Results
	A. 50 Micrograms of Estrone Daily (3 Cancers at 550-598 Days)				
1	F	1 mo.	No	284	Cystic changes
2	M	1 mo.	Yes	550	Cancer
3	M	1 mo.	Yes	591	Cancer
4	M	1 mo.	Yes	598	Cancer
	B. 100 Micrograms of Estrone Daily (4 Cancers at 359-408 Days)				
1	M	1 mo.	Yes	29	Cystic change
2	F	1 mo.	Yes	37	Cystic change
3	M	1 mo.	Yes	359	Cancer
4	F	1 mo.	Yes	378	Cancer
5	M	1 mo.	Yes	387	Cancer
6	M	1 mo.	Yes	408	Cancer
	C. 200 Micrograms of Estrone Daily (8 Cancers at 137-281 Days and 1 Cancer at 334 Days)				
1	F	1 mo.	Yes	174	Cystic change
2	F	1 mo.	Yes	188	Cancer
3	F	1 mo.	Yes	150	Cancer
4	F	1 mo.	Yes	334	Cancer
5	M	1 mo.	No	300	Cystic change
6	M	1 mo.	No	300	Atrophy
7	M	1 mo.	No	281	Papillary cancer
8	M	1 mo.	No	300	Cystic change
9	M	1 mo.	No	194	Duct cancer
10	M	1 mo.	No	300	Cystic change
11	F	1 mo.	No	234	Atrophy
12	F	1 mo.	No	260	Cystic change
13	F	1 mo.	No	137	Duct cancer
14	F	1 mo.	No	197	Cystic change
15	F	1 mo.	No	224	Lobular and duct cancer
16	F	1 mo.	No	238	Duct cancer
17	F	1 mo.	No	224	Cystic change
D. 100 Micrograms of Stilbestrol Daily (8 Cancers at 104-222 Days. Compare with B)					
1	M	2 mo.	No	92	Cysts, fibrosis and atrophy
2	F	2 mo.	Yes	104*	Cancer
3	F	2 mo.	Yes	163	Sweat gland adenoma
4	F	2 mo.	Yes	202	Cancer
5	M	2 mo.	No	222	Cancer
6	F	1½ mo.	No	130	Duct cancer; very early comedo cancer
7	F	1½ mo.	No	130	Small comedo duct cancer
8	F	1½ mo.	No	130	Duct cancer
9	F	1½ mo.	No	130	Very early comedo cancer
10	F	1½ mo.	No	130	Very early lobular cancer

* This rat received 200 micrograms daily.

are formed. These cysts appear within twenty to forty days and are accompanied by secretory changes in the epithelium. The cysts are preceded by increase in the size and the number of the epithelial buds at the ends of the tubules. The cysts have no direct relation to the subsequent formation of cancer and are associated with rapid ripening and secretion in the epithelium of the terminal tubules (fig. 2*A*). When cysts form,

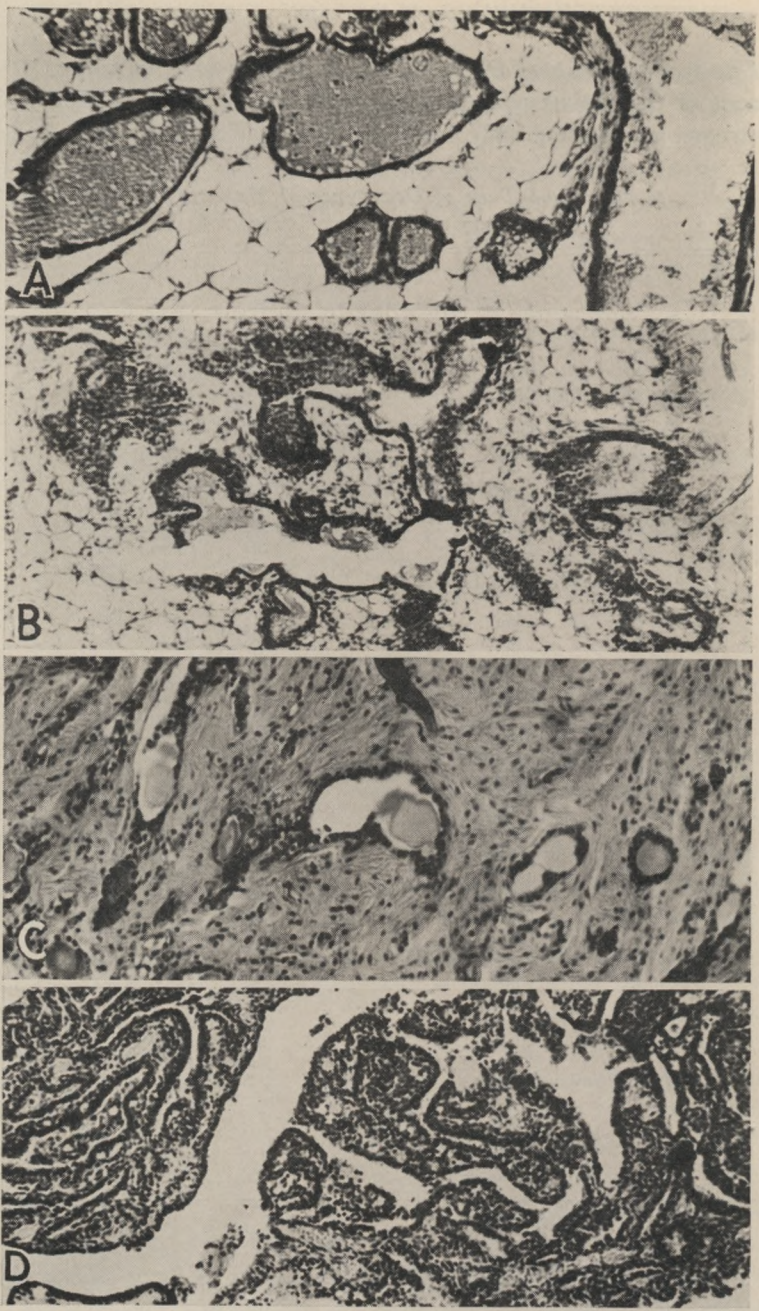

Fig. 2.—Pathologic changes in the mammary gland preceding the development of estrogenic mammary cancer: *A*, large cysts occurring after daily injections of estrone in oil; *B*, adenosis and epithelial hyperplasia following implantation of pellets; *C*, fibroadenoma following implantation of pellets; *D*, benign papilloma following implantation of pellets.

the appearance of the cancer is delayed, and a more prolonged estrogenic effect with continuous epithelial regeneration followed by degeneration and atrophy must occur before cancer appears. This corresponds with

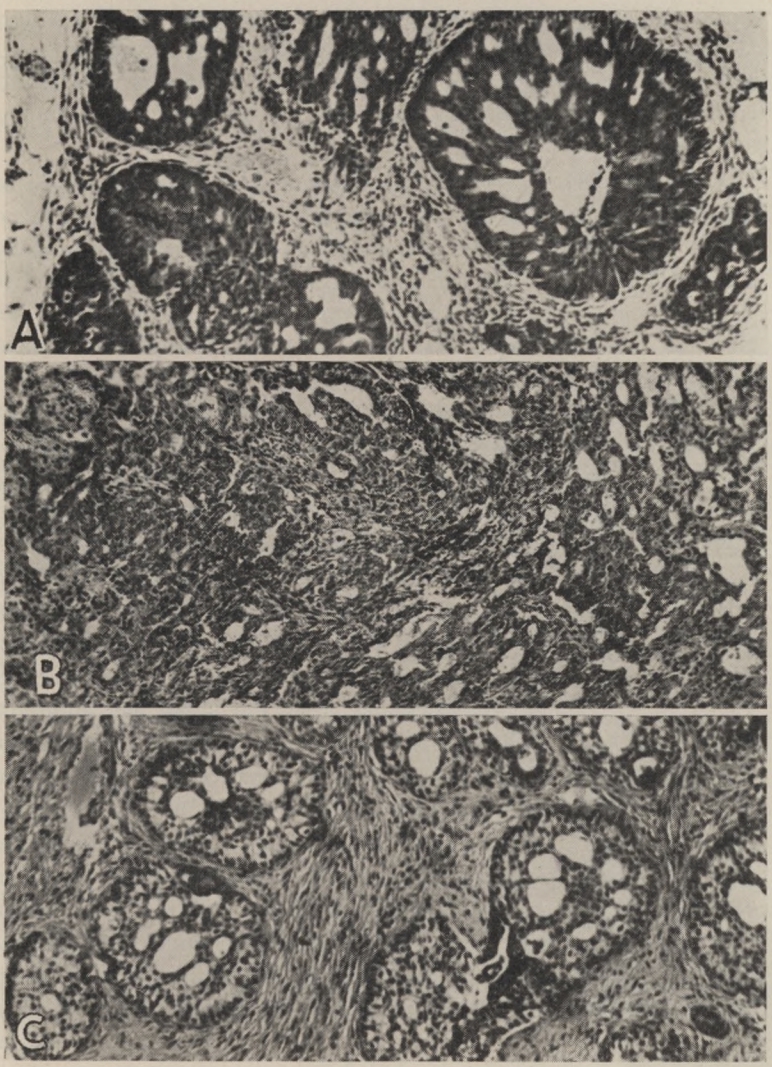

Fig. 3.—Varying forms of mammary carcinoma with different methods of dosage: *A*, comedo cancer obtained with interrupted daily doses; *B*, lobular cancer obtained by implantation of pellets; *C*, duct cancer obtained by implantation of pellets.

observations in the human breast which indicate that cystic disease occurring in women toward the menopause is not associated (except

coincidentally) with mammary cancer. It is usually a self-limited disease and associated with a relatively brief period of estrogenic overstimulation at or near the menopause.

With intense interrupted estrogenic stimulation (daily injections of estrone in oil) in castrate female rats, lobule formation does not occur in the breast, and as a result the cancers formed are confined to the ducts and terminal tubules, and their pathologic variety is thus limited. Under these conditions comedo cancer is the predominant pathologic form (fig. 3).

Effect of Continuous Absorption (Implantation of Pellets).—When an estrogen is injected in oil, absorption is rapid for the first several hours, but soon thereafter the concentration in the tissues diminishes. Under these conditions there are daily variations in the estrogenic level. The agent is more effective if the concentration is maintained by more gradual and constant absorption, as from pellets [5] implanted beneath the skin of the back.

Mammary cancer in the rat occurs with smaller amounts of estrogen when pellets of the agent are implanted (tables 3 and 4). As little as 9 to 10 mg. of estrone in the form of two to three pellets given individually at intervals will produce cancer in two hundred and fifty to three hundred days. Although when pellets are used mammary cancers are produced with less estrogen than when injections are used, not all the animals show the same rate of development of cancer, and cancer does not appear in all of them. In young animals benign tumors (papilloma or fibroadenoma) may first appear (fig. 2). Later, cancer may occur in the benign fibroadenoma, after the latter has undergone involutional changes. When estrone pellets are used in castrated male rats, most of them die within two to three months from urinary obstruction caused by epidermal thickening in the urethra.

The formation of fibroadenoma in response to estrone from pellets is due to the selective action of moderate, continuous and prolonged estrogenic stimulation on the connective tissue. The fibrous stroma of the breast is apparently more susceptible to such moderate and prolonged stimulation than to the short intense stimulation of high doses. The fibrosis occurring in the mammary gland, therefore, serves as an index of the duration and the intensity of the estrogenic stimulation. When it is most marked, the stimulation has been moderate, continuous and of long duration.

5. These pellets are small plugs of crystalline estrogen, weighing between 2.5 and 3.0 mg. They are made by tightly compacting the crystals in drilled holes in a steel plate. The compactness of the pellet is such that absorption takes place only from the surface, slowly and continuously.

If the ovaries are present when pellets are implanted, the slow continuous absorption of the estrogen results in more prolonged and increased luteinization of the ovaries and in mammary lobule formation approaching that seen in the breast in pregnancy. Even in the absence of the ovaries estrone from the pellets stimulates moderate, irregular lobule formation. The lobulation is significant, since it influences the

TABLE 3.—*Mammary Cancers Produced in Rats by Implantation of Estrone Pellets Weighing 2.5 mg. Each*

Rats	Sex	Total Dose, Mg.	Day of Life on Which Pellets Were Implanted	Tumors Produced	Days Required to Produce Cancer
7	F	5.5 †	2d 28th 153d	Cancer * in 4; benign fibroadenoma only in 2 (135 and 190 days)	255 to 350 days *
5	M	5.5	2d 28th 153d	Cancers in 2; fibroadenoma in 0	172 to 300 days

* One animal with four small pellets implanted on the twenty-third day of life had microscopic cancer on the forty-fourth day of life; other microscopic cancers appeared from one hundred and seventy-two to three hundred and fifty days after the starting of treatment. In 2 of the 4 females in which cancer developed, benign fibroadenoma of the breast developed preceding cancer. In 2 additional females, benign fibroadenoma only developed.

† Since the pellets were not completely absorbed, the dose was less than the total weight of the pellets (7.5 mg.). These rats were not castrated.

TABLE 4.—*Early Cancer with Multiple Pellets—Total Dose 12.5 mg.*

Rat	Age	Sex	Castrated	Estrogen	Days Required for Cancer
1	21 days	F	Yes	Estrone	21
2	26 days	F	Yes	Estrone	25
3	21 days	F	Yes	Estrone	30
4	21 mo.	F	No	Estrone	47
5	21 mo.	F	No	Estrone	60
6	21 mo.	F	No	Estradiol	47
7	60 days	F	Yes	Estrone	60
	15 mo.	F	No	Stilbestrol	35
8	9 mo.	F	Yes	Stilbestrol	90

pathologic form of the mammary cancer. Under these conditions infiltrating lobular cancers may form (fig. 3).

The more gradual and continuous absorption of an estrogen from implanted pellets results in a more widespread and extensive epithelial response. Epithelial proliferation may for a period of months predominate over epithelial ripening and secretion. Adenosis with formation of duct adenoma and intraductal papilloma occurs in addition to fibroadenoma and precedes or replaces the formation of large cysts

(fig. 2). Again the epithelial changes are not in themselves precancerous. This is proved by their gradual involution and disappearance if the pellets are removed (or by the absence of cancer if the initial low concentration of estrogen is maintained by daily injection of physiologic amounts of the agent throughout the life of the animal in the absence of pellets). However, once these epithelial changes have been produced, the mammary gland is more susceptible to cancer *provided a steadily increasing concentration of estrogen is maintained* by allowing the pellets to remain and by implanting additional ones.

The modified action of estrogen administered by implantation of pellets instead of by injection is also apparent when stilbestrol or estradiol is administered. With daily injections of 100 micrograms

TABLE 5.—*Comparison of Doses and Periods of Treatment Required with Different Estrogenic Compounds*

Compound	Dose, Micrograms	Total Amount, Mg.	Days Required to Produce Cancer
Estradiol (injected)	100	25	290
Estradiol (implanted)	3 successive pellets	8	310
Estradiol dipropionate	5	0.6	120
Estradiol benzoate	5	0.6	120
Stilbestrol (injected)	100	15.0	150
Stilbestrol (implanted)	5 successive pellets	10.1	105
Stilbestrol monomethyl ether	50	9.5	212
Stilbestrol dimethyl ether *	500	116.5	233

* The cancer-producing efficiency of estrogen derivatives with a prolonged action is not apparent when the estrogenic potency of the derivative is far below that of the parent compound (table 1).

of stilbestrol, 18 mg. of the substance is necessary for the production of mammary cancer, whereas only 10 mg. is required if pellets are used. With daily injections of 100 micrograms of estradiol in oil, 25 mg. is required to produce cancer; with implantation of pellets 8 mg. will suffice. The more continuous estrogenic action observed with stilbestrol and estradiol pellets is associated with the formation of fibroadenoma, adenosis and other changes similar to those described in the animals receiving estrone pellets. In general, the total amount of the estrogen necessary to produce cancer may be decreased to one-half or to one-third if the agent is implanted in the form of pellets rather than injected in oil (table 5).

Instead of estrone, estradiol or stilbestrol pellets, estrogenic compounds with a prolonged action may be used. The tissue changes are similar to those produced by the implantation of pellets.

Estradiol compounds in which a chemical radical is introduced to prolong estrogenic action are more efficient in producing cancer than the original compound even though the estrogenic potency has been lessened somewhat by the addition of the radical. This is also true of the ethers of stilbestrol which have a prolonged action.

Table 5 gives a comparison of the amount of the agent and the time required for production of cancer with derivatives of estradiol injected in oil as compared with the original compound administered by injection or in the form of pellets. Table 5 also gives the corresponding information for stilbestrol and its derivatives.

Spontaneous mammary cancer is rare in the rat and is difficult to maintain by transplantation. Estrogenic mammary cancer in the rat, therefore, provides valuable experimental material for the study of the

TABLE 6.—*Percentage of Rats in Which Mammary Cancer Developed When Varying Amounts of Estradiol Benzoate in Oil Were Injected Daily*

Rats *	Age at Onset of Treatment	Dose, Micrograms	Duration of Treatment	Cancers	Percentage
5	2 mo.	5	120 days	5	100
5	2 mo.	10	120 days	5	100
12	7 mo.	50	120 days	6	50
12	1½ mo.	100	120 days	9	75
8	6 mo.	100	120 days	5	62
22	7-9 mo.	100	120 days	11 †	50
6	1½ mo.	200	120 days	4	67
70				45	

* The numbers include both males and females; none of the rats were castrated.
† The majority of these cancers occurred in eighty to one hundred days.

disease. These cancers can be produced at an early age and relatively quickly if the proper estrogen and mode of administration are used. In our experience, cancers are produced most quickly and in a high percentage of the animals if 5 micrograms of estradiol benzoate is injected daily except Sundays in rats from 1 to 3 months of age. With this dose, the tumors appear in microscopic form in one hundred and twenty days, and palpable nodules appear shortly thereafter in one hundred and twenty-five to one hundred and seventy days. When more than 5 micrograms of estradiol benzoate is given daily, the cancers appear at the same rate, and the percentage of rats in which cancer develops is not increased but tends to decrease.[6]

6. With the daily injection of 100 micrograms of stilbestrol in oil, cancer appeared in approximately 100 per cent of the rats in one hundred and forty to one hundred and fifty days.

EFFECT OF PROLONGED REGENERATION

Since the susceptibility to cancer is influenced by the capacity for regeneration in the tissue the preexisting physiologic state of the gland is important. If epithelial regeneration below the cancer-producing level has been previously stimulated in the mammary gland, it should be possible at some later date to produce cancer more readily.

In a group of rats 1 month old, intense estrogenic stimulation (200 micrograms of estrone) was given for ninety days. Biopsy showed that no cancers had developed. These rats were allowed to live one and a half years without further treatment, and at the end of this time short intense estrogenic stimulation was given. Cancer appeared quickly in from two to three months. In another group of rats physiologic doses of estrone (2.5 to 5 micrograms) were given daily for a period of almost two years, and no cancers appeared. In these animals at the end of this time cancer developed within two months after the injection of large daily doses of estrone. Thus, the effects of a previously intense or prolonged period of estrogenic stimulation may persist as a latent cancerogenic influence in the breast (tables 7 and 8).

Because of the latent predisposition to cancer in the breasts of these animals, the appearance of these glands under the microscope before the final period of estrogenic stimulation is important. In the group that had intense stimulation for ninety days in early life, the glands were markedly atrophic and nearly replaced by fat. Their structure resembled the so-called "senile, fatty breast" so often described in pathologic reports on human breasts removed for cancer.

In the group of rats which had been castrated and which received daily injections of estrone in doses within physiologic limits (2.5 to 5 micrograms), the mammary glands were markedly fibrosed and the terminal tubules showed irregular epithelial proliferation resembling adenosis or Schimmelbusch's disease in the human breast (fig. 2 *B*). It is significant that women with adenosis when observed at intervals through a period of years (and past the menopause) show a greater tendency toward mammary cancer than those with normal breasts in the same age groups.

It is in these last two experiments in which preexisting changes in the breast (resulting from previous estrogenic stimulation) combine with the effects of a final period of intense estrogenic action to produce cancer that conditions found in the human breast with cancer are most closely approximated. These experiments suggest that human mammary cancer may result from one or a combination of the following factors:

1. Abnormally intense estrogenic stimulation during the adolescent period of mammary development or during a previous pregnancy.

(Some of these patients will recall varying degrees of virginal or gravid hypertrophy.)

2. Ovarian dysfunction in cyclic women resulting in relative hyperestrogenism over a period of years prior to the menopause. (Most of

TABLE 7.—*Cancer Produced in Castrated Rats by Administration of Initial Massive Doses of Estrone at the Age of 1 Month Followed by an Interval of No Treatment, Then by Further Administration of Massive Doses*

Sex of Rat Used	Initial Dose: Amount, Micrograms	Initial Dose: Duration, Days	Interval Without Treatment, Days	Final Dose: Amount, Micrograms	Final Dose: Duration, Days	Microscopic Picture: Days After Initial Dose	Microscopic Picture: Days After Final Dose
F	200	90	563	2 pellets into breast	89	338, fibrosis 522, infected nipple 620, atrophy 652, atrophy	23, hyperplasia 89, cancer
M	200	90	563	2 pellets into breast	54	144, fibrosis 565, fibrosis, irregular lobules 652, atrophy	23, hyperplasia 54, cancer
M	200	90	563	2 pellets into breast	54	565, fibrosis 652, atrophy	23. hyperplasia 54, cancer
F	200	90	754	None, control	..	216, fibrosis 620, fibrosis 675, atrophy 741, senile atrophy	90, no change
M	200	90	...	None, control	..	620, atrophy 675, atrophy	90, no change

TABLE 8.—*Cancer Produced in Castrated Rats by Administration of Large Doses of Estrone Following Prolonged Administration of Physiologic Doses Begun at the Age of 1 Month*

Sex of Rat Used	Physiologic Dose: Amount, Micrograms	Physiologic Dose: Duration, Days	Massive Dose: Amount, Micrograms	Massive Dose: Duration, Days	Microscopic Picture: 594 Days After Physiologic Dose	Microscopic Picture: Days After Massive Dose
M	2.5	594	200	98	Irregular lobules and moderate fibrosis	10, negative for cancer
M	2.5	594	200	98	Cystic dilatation, some adenomatous lobules	23, fibroadenoma 41, precancerous changes 98, comedo cancer
F	5.0	594	200 Pellet, 3 mg. 200	11 13 74 98	Cysts and lactating adenoma	11, atrophy 23, comedo cancer 41, duct cancer 68. gross cancer 98, large cancer and fibroadenoma
F	5.0	594	200 Pellet, 3 mg. 200	11 13 74 98	Fibrosis and fibroadenoma	11, fibroadenoma 41, comedo cancer 98, gross cancer

these patients have the characteristic changes of adenosis or Schimmelbusch's disease.)

3. Intense or continuous estrogenic stimulation occurring at the time of menopause (superimposed on the factors enumerated under 1 and 2).

EFFECT OF AGE

According to vital statistics, the incidence of human mammary cancer increases steadily with the age of the population. The atrophy and the degenerative changes found in the senile breast may therefore be etiologic factors in mammary cancer. With respect to estrogenic mammary cancer in the rat, histologic studies indicate that premature ripening and accelerated regeneration which terminate in degenerative changes may be precursors of cancer. In line with this interpretation a comparison was made of the periods required for the appearance of cancer in young and old rats.

It was found that the time required for the appearance of cancer was inversely proportional to the age of the animal in a series in which

TABLE 9.—*Time Required to Produce Mammary Cancer with Multiple Estrone Pellets in Rats of Varying Ages*

Rats	Age	Sex	Number of Pellets Administered	Cancers	Time Required, Days	Average Number of Days
30	1 wk.	F	One at a time	17	105-532	300
10	1 mo.	Both*	Multiple	4 2	175-385 21-25	293
10	2½ mo.	F	Multiple	5 1	175-317 26	248
10	4 mo.	Both	Multiple	6 1	101-399 35	248
6	7½ mo.	Both	Multiple	3 1	165-255 31	205
4	9 mo.	F	Multiple	2	180-210	195
8	20 mo.	F	Multiple	4 2	64-109 27-48	90

* The group was made up of males and females in equal numbers. Cancer developed in both sexes with equal frequency.

estrone pellets were implanted, but there were a certain number of exceptions in which cancers appeared early regardless of the age (table 9). With other methods of estrogenic stimulation, no significant difference between young and mature rats was found. Thus, as shown in table 10, the majority of the mammary cancers were observed within one hundred and twenty days regardless of whether the injections (100 micrograms of estradiol benzoate) were begun at the age of 1½, 6 or 11 months. A certain number of early cancers occurred in all of the experimental animals in which continuous absorption of the estrogen took place, particularly if multiple pellets were inserted. These early cancers developed within twenty-one to ninety days after treatment and were dependent on the method of dosage and were independent of the age of the animals (table 4). When the data were summarized for all methods of estrogenic stimulation, the chronologic age was found to be

without significance for the time of appearance of the mammary cancers. Additional experiments, utilizing rats aged 20 months or more, are being carried out since an insufficient number at this age have been tested.

EFFECT OF OTHER SEX HORMONES

Injections of either progesterone or testosterone over a long period alone will cause increased lobular development in the mammary gland of the rat whether the animal is castrate or noncastrate, male or female.

TABLE 10.—*Results with Administration of 100 Micrograms of Estradiol Benzoate*

Rats Used	Age of Rats When Treatment Started	Results of Biopsy		Cancers at Autopsy After 120 Days of Treatment
		After 78 Days of Treatment	After 105 Days of Treatment	
6 Females	1½ mo.	.....	1 cancer	5
6 Males	1½ mo.	2 cancers	.....	4
4 Females	6 mo.	.....	Fibroadenoma only	2
4 Males	6 mo.	.....	Adenosis and cysts only	3
2 Females	11 mo.	.....	Adenosis only	1

TABLE 11.—*Mammary Cancer in Rats Receiving Testosterone and Estrone*

Rat Used	Dosage of Testosterone	Dosage of Estrone	Result
Female castrate	0.5 mg. 1-18 days	100 micrograms, 30-57 days 200 micrograms, 57-162 days	Autopsy after 162 days: cancer and adenosis
Female castrate	0.5 mg., 73-108 days 0.5 mg., 143-180 days	200 micrograms, 31-72 days 200 micrograms, 108-143 days	Autopsy after 192 days: comedo cancer
Male castrate	0.5 mg., 285-357 days	25 micrograms, 25-285 days 50 micrograms, 357-407 days Estrone pellet, 407th day, 493d day	Autopsy after 530 days: cancer, perithelioma-tous type
Female castrate	0.115 mg., 80-249 days 1.25 mg., 249-266 days	10 micrograms, 80-249 days	Comedo cancer after 266 days of treatment
Female castrate	0.25 mg., 79-249 days	20 micrograms, 79-249 days	Duct cancer after 99 days of treatment
Male intact	1 pellet	5 pellets	Cancer after 383 days
Female intact	0.5 mg., 43-73 days*	5 pellets	Comedo and lobular cancer after 73 days

* Progesterone was substituted.

Testosterone is more efficacious than progesterone in producing lobular growth. No cancers were observed in the breasts when injections of progesterone alone were given over a period of several months or when testosterone was given over a period of more than a year; the only significant mammary change was a lobular formation resembling that of midpregnancy. However, pathologic changes and cancer formation occurred if injections of testosterone or progesterone were given simultaneously with injections of estrone or when a similar combination of pellets was given (table 11). Under such endocrine stimulation the formation of large mammary cysts was prevented. The degenerative changes preceding cancer appeared in the acinous structures and

resembled postlactation involution. The earlier changes resulting from combined hormone stimulation were characterized by irregular epithelial proliferation resembling that seen in the human breast with adenosis. It is significant that the addition of testosterone or of progesterone did not prevent the occurrence of mammary cancer in these experiments. Instead, in some animals lobular cancers were apparently the result of stimulation by these hormones, since the amount of estrogen used was insufficient by itself to produce the cancers. The gonad-stimulating and the lactogenic hormones of the anterior lobe of the pituitary gland which produce lobule formation and secretion in the mammary gland of intact animals will, however, inhibit the growth of estrogenic mammary cancer. This is discussed subsequently.

CHANGES IN THE ENDOCRINE ORGANS IN THE EXPERIMENTAL PRODUCTION OF MAMMARY CANCER

Pathologic changes in the endocrine organs have been reported by most of the workers who have produced mammary cancer with high doses of estrogens in mice (Cramer and Horning [7]). The pituitary gland enlarges because of an increase in the number of chromophobe cells in its anterior lobe. Such changes were found at autopsy in the animals in the present experiments (fig. 4). In addition these animals showed degenerative changes in the gonads with formation of benign cysts or papillomatous cysts of the ovary (2 animals had granulosa cell cancer, another metastasizing adenocarcinoma and another mesonephroma of the ovary). There was enlargement of the adrenal cortex, and occasionally there were degenerative changes in the medulla. Cystic degeneration occurs in the thymus gland with compensatory hypertrophy of the mediastinal lymph nodes, which may terminate in lymphosarcoma or leukemia (table 12).

The most striking and consistent changes are in the pituitary gland and in the thymus. The increase in the number of chromophobe cells in the anterior lobe of the pituitary leads to hypertrophy of adenomatous proportions. Most of the experimental animals have glands five or more times normal size, and hemorrhage may occur in the adenomatous tissue (fig. 4). The significance of these changes in the development of mammary cancer is not established. Hypophysectomy prevents the physiologic effects of estrogen on the mammary gland, and no cancers develop. The importance of the pituitary gland for the development and normal physiologic response of the breast has been stressed by other workers (Gomez and Turner [8]). It has also been noted in the present

7. Cramer, W., and Horning, E. S.: Lancet **1**:247, 1936.

8. Gomez, E. T., and Turner, E. W.: Hypophysectomy and Replacement Therapy in Relation to the Growth and Secretory Activity of the Mammary Gland, Research Bulletin 259, University of Missouri College of Agriculture, Agricultural Experiment Station, 1937.

group of experiments. Estrogen response was prevented by hypophysectomy (Astwood, Geschickter, and Rausch [9]). Hypophysectomy prevented the appearance of cancer even in those animals which lived the required length of time (six months or more) and in which the removal of the pituitary was apparently incomplete.[10]

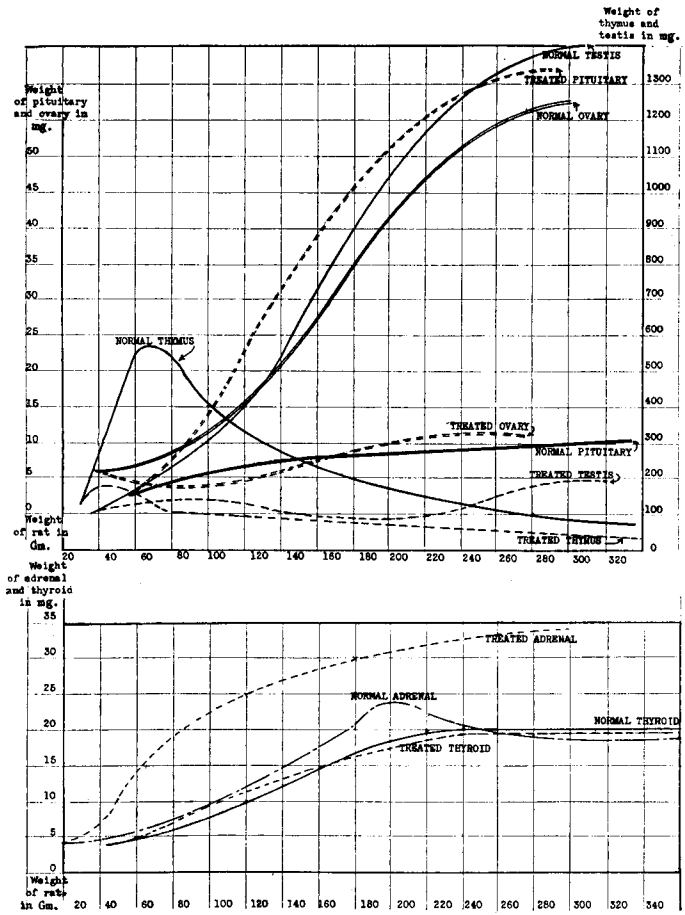

Fig. 4.—Effect of developing estrogenic mammary cancer on the endocrine glands of rats: Upper graph, effect of prolonged administration of estrogens on the weights of the pituitary, the thymus and the gonads; lower graph, effect on the weights of the adrenal and the thyroid gland.

9. Astwood, E. B.; Geschickter, C. F., and Rausch, E. O.: Am. J. Anat. **61**:373, 1937.

10. The hypophysectomized animals used in the experiments were prepared by Dr. R. O. Greep of the Squibb Institute for Medical Research.

Striking involutional changes occur in the thymus glands of rats maintained under continuous overstimulation with estrogen. The glands shrink in size, and the thymic tissue is replaced by cysts lined with transitional epithelium resembling that seen during the embryologic development of the thymus and the thyroid. This thymic involution is

TABLE 12.—*Lymphosarcoma and Myeloid Leukemia in Rats Treated with Estrogens*

Rat	Age	Sex	Treatment	Days Treated	Results
1	1 mo.	F	2 hr. radium (30 mg.) over thymus; 100 mg. estradiol benzoate	225	Lymphosarcoma; cancer of breast
2	1½ mo.	F	2 hr. radium (30 mg.) over thymus; 100 mg. estradiol benzoate	229	Lymphosarcoma
3	1½ mo.	F	2 hr. radium (30 mg.) over thymus; 100 mg. estradiol benzoate	210	Lymphosarcoma; cancer of breast
4	3½ mo.	F	5 hr. radium (30 mg.) to mediastinum; 100 mg. estradiol benzoate	145	Lymphosarcoma; cancer of breast
5	3½ mo.	F	6 hr. radium (30 mg.) to mediastinum; 100 mg. estradiol benzoate	165	Lymphosarcoma; cancer of breast
6	4 mo.	F	6 hr. radium (30 mg.) to mediastinum; 100 mg. estradiol benzoate	150	Lymphosarçoma; cancer of breast
7	20 mo.	F	100 mg. estradiol benzoate daily	690	Lymphosarcoma of spleen; cancer in fibroadenoma of breast
8	1 wk.	M	3 successive estrone pellets.....	150	Myeloid leukemia
9	1 wk.	F	3 successive estrone pellets.....	420	Cancer of breast: lymphosarcoma of thymus
10	1 wk.	F	3 successive estrone pellets.....	420	Lymphosarcoma of thymus and spleen; cancer of breast
11	1 wk.	F	3 successive estrone pellets.....	480	Lymphosarcoma of spleen; cancer of breast
12	1 wk.	F	3 successive estrone pellets.....	360	Lymphosarcoma of thymus and mesenteric nodes; cancer of breast
13	1 wk.	M	3 successive estrone pellets.....	420	Lymphosarcoma of thymus; cancer of breast
14	1 mo.	M	5 estrone pellets.....	300	Lymphosarcoma of breast; beginning cancer of breast
15	13 mo.	F	6 stilbestrol pellets—repeated in four months	165	Lymphosarcoma; cancer of breast
16	21 mo.	M	5 estrone pellets.....	60	Lymphosarcoma

marked after prolonged administration of estrogen but also occurs when other sterol hormones, e. g., testosterone and progesterone, are administered. Whether or not it plays a role in the development of mammary cancer is being investigated further.

Changes in the adrenal glands are most frequently found in the reticular zone of the cortical tissue. The size of the cells and their lipid contents are increased. The result is a moderate increase in the size and

the weight of these glands in the animals receiving estrogens and in those in which mammary cancer develops. The changes in the adrenal medulla are variable. Degeneration and calcification are occasionally seen, but most often the appearance of this tissue is normal.

No significant changes were found in the thyroid glands of the treated animals (fig. 4 *B*), although marked changes with squamous cell metaplasia have been reported in response to estrogens by other workers (Mark and Biskind [11]).

The atrophic changes in the gonads are probably significant in the development of mammary cancer. There were no apparent differences in percentage of cancers or in time of their appearance between castrate and noncastrate rats whether these were males or females, but this may be explained by the atrophy of the gonads when estrogen is administered in large amounts.

The size of the ovaries may be reduced from a diameter of 5 to 8 mm. in the normal adult to a millimeter or less in those animals which have had prolonged treatment with estrogen. In the atrophic ovaries, there is a decrease in the number of follicles, and those persisting are decreased in size or undergo cystic degeneration. Luteal changes are absent. The interstitial cells are increased in number but are small and compact. Cysts with intracystic papillomas are occasionally observed. In 4 animals malignant ovarian tumors occurred. One of these was a metastasizing adenocarcinoma; another was a small and malignant mesonephroma (fig. 5). In the animal in which adenocarcinoma of the ovary developed, squamous cell cancer of the cervix was also present, as well as mammary carcinoma. This was the only animal with a definitely malignant tumor of the cervix, although there was 1 other with early malignant change in the cervix.

After prolonged treatment with estrogens, the size of the testicle is reduced, the spermatic tubules are small and collapsed, and spermatogenesis is absent. Tumor formation in the testicle has not been observed.

The early effect of estrogenic stimulation of the prostate is an increase in the thickness of the lining epithelium in the prostatic urethra, which becomes increasingly keratinized. The fibromuscular stroma of the prostate is hypertrophied, and the glandular epithelium shows increase in the size of the cells and occasional dilation of acini. Later the fibromuscular stroma practically obliterates the glandular elements and the prostate shrinks in size. Cornification extends into the suburethral glands of the prostatic urethra, and there may be an irregular downgrowth of cells suggesting beginning transitional cell carcinoma in the prostatic urethra. Squamous cell cancer at this site or adenocarcinoma of the prostate has not been observed.

11. Mark, J., and Biskind, G. R.: Endocrinology **28**:465, 1941.

The thickening of the prostatic urethra leads to urinary obstruction. The ureters become dilated and thickened, and the bladder is also markedly distended and increased in size and thickness.

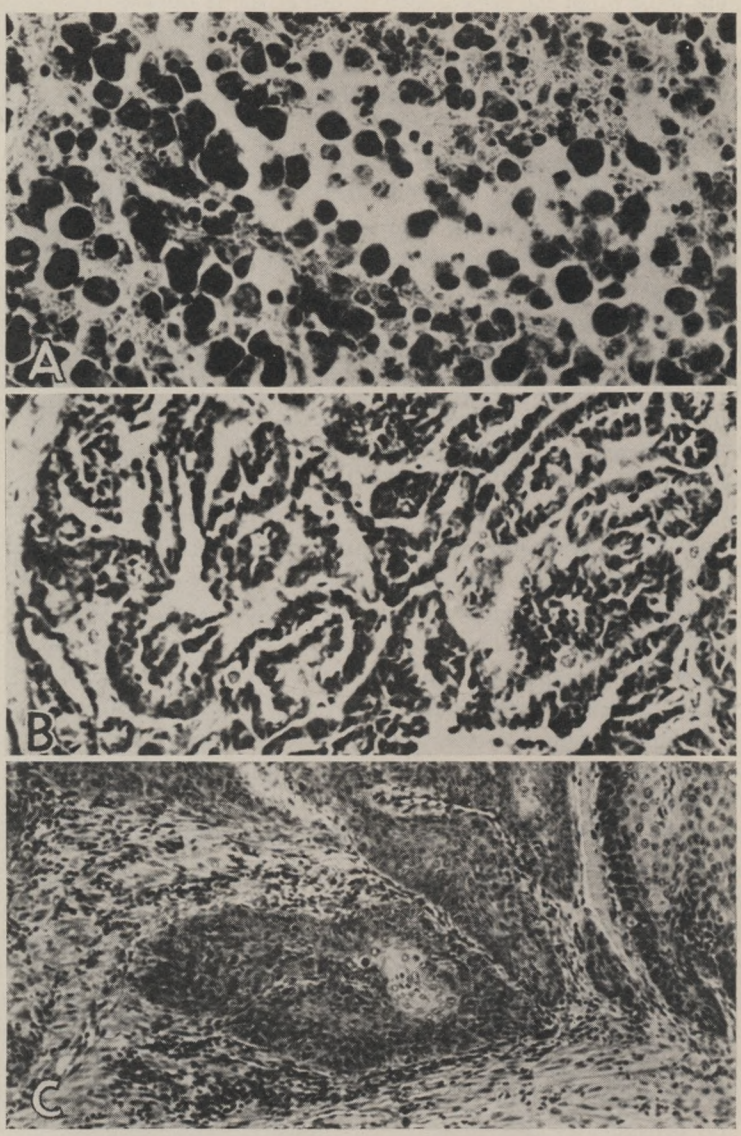

Fig. 5.—Cancers associated with the development of estrogenic mammary cancer in the rat: *A*, lymphosarcoma; *B*, cancer of the ovary; *C*, cancer of the cervix.

In the female genital tract keratinization and thickening occur in the mucous membrane of the vagina and cervix. These changes extend into the mucous glands and into the uterus, replacing the normal endo-

metrium. These changes are akin to those observed in the prostatic urethra. Carcinoma of the cervix arising at the base of the thickened cornified epithelium was found in 2 cases. Adenocarcinoma of the uterus was not observed.

Some workers (Cramer and Horning[7]) have sought to show that estrogenic cancer of the mammary gland is the result of changes in the endocrine system as a whole, particularly in the pituitary body and the adrenal glands, rather than a direct effect on the breast. These authors thought that they could inhibit mammary cancer by injecting a pituitary extract containing the thyrotropic factor. These results could not be confirmed by Asdell and Seidenstein[12] or by Lacassagne.[13] While we have been able to demonstrate that the presence of pituitary gland is necessary for the production of estrogenic mammary cancer, we have insufficient evidence to prove that the estrogenic effect on the mammary gland is an indirect one.

One of our experiments indicates that the growth of estrogenic mammary cancer may be partially inhibited by injections of anterior pituitary extracts. A group of 12 rats, 1 month old, received 10 micrograms of estradiol benzoate daily for a period of six months. At the end of one hundred days one third of the animals were autopsied and, on microscopic study of breast tissue, all of them were found to have mammary cancer. The remainder of the animals continued to receive their daily injections of the estrogen. In all but 2, which were reserved for controls, daily injections of anterior pituitary extract were added for the next four months. (These daily injections consisted of 5 rat units of a follicle-stimulating extract and 20 guinea pig units of a preparation containing the thyrotropic factor [ambinon, Roche-Organon] daily for one month followed by 10 international units of prolactin [Schering Corporation] daily for one month. Thereafter, these two cycles of injections of anterior pituitary extracts were each repeated for one month to complete the four months of treatment.) Palpable cancers developed in the animals which received only injections of estradiol benzoate. However, in the animals receiving in addition injections of anterior pituitary preparations palpable cancers were not found at autopsy, but all except 1 of the animals had microscopic cancers. Cramer,[14] who used injections of ambinon only, believed that the inhibiting effect of this anterior pituitary extract was more marked than indicated in this experiment.

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12. Asdell, S. A., and Seidenstein, H. R.: Proc. Soc. Exper. Biol. & Med. **32**:931, 1935.

13. Lacassagne, A.: Am. J. Cancer **37**:414, 1939.

14. Cramer, W.: Am. J. Cancer **38**:463, 1940; Lancet **1**:192, 1939.

SUMMARY

All of the estrogens of sufficient potency for clinical use will produce mammary cancer in the rat regardless of chemical composition or physiologic potency. To produce mammary cancer, the dose must be well beyond the physiologic limit (ten or more times the threshold dose) and the treatment continuously applied for a period of months (thirty or more times the duration of normal estrus).

Variation in the time required to produce mammary cancer is dependent on: dosage, estrogenic potency, duration of estrogenic activity and method of administration. The total dose required to produce mammary cancer is not influenced by the amount of the daily dose but varies with the duration of estrogenic activity and the method of administration. It is difficult to demonstrate that sex, age or castration influences susceptibility to estrogenic mammary cancer. An initial period of estrogenic stimulation below the cancer-producing threshold renders the gland more susceptible to estrogenic cancer.

Important species differences are evident on the basis of experiments carried out in monkeys, rabbits and rats. A case of estrogenic mammary cancer in the rabbit is recorded.

Administration of testosterone or progesterone simultaneously with, or in sequence to, estrogenic stimulation does not prevent the appearance of mammary cancer. However, the growth of estrogenic cancer is inhibited by anterior pituitary extract.

To date, from a toxicologic standpoint the most important finding with respect to the estrogens is the production of mammary cancer by prolonged administration. In the clinical administration of the estrogens this toxicologic property of these compounds has not yet been verified but must be considered when compounds of high potency with prolonged activity are administered. The species difference remains an unknown factor.

Various changes in the endocrine glands accompany the appearance of mammary cancer, and cancerous change has been observed in other organs.

Preparations for use in this study were supplied by the manufacturers as follows: testosterone propionate, progesterone and estradiol dipropionate by the Ciba Pharmaceutical Products, Inc.; estradiol benzoate and a preparation of the anterior pituitary lactogenic factor (prolactin) by the Schering Corporation; an anterior pituitary extract containing the thyrotropic factor (ambinon) and estrone by Roche-Organon, Inc.; stilbestrol, stilbestrol dimethyl ether and stilbestrol monomethyl ether by Wallace & Tiernan Products, Inc.